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Hohl, M ; Christensen, O ; Kunz, C ; Naegeli, H ; Fleck, O

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Marcel Hohl, Olaf Christensen, Christophe
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Binding and Repair of Mismatched DNA Mediated by Rhp14 the Fission Yeast Homologue of Human XPA*

Marcel Hohl†‡§, Olaf Christensen‡||§, Christophe Kunz‡, Hanspeter Naegeli¶, and Oliver Fleck‡**

‡Institute of Cell Biology, University of Bern, Baltzerstrasse 4, CH-3012 Bern, Switzerland

¶Institute of Pharmacology and Toxicology, University of Zürich-Tierspital, August Forel-Strasse 1, CH-8008 Zürich, Switzerland

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§ These authors contributed equally to this work.

† Present address: Institute of Medical Radiobiology, University of Zürich, August Forel Strasse 7, CH-8008 Zürich, Switzerland.

|| Present address: Institute of Microbiology, ETH-Zürich, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland.

** To whom correspondence should be addressed: Oliver Fleck; Tel.: 41-31-631-4656; Fax: 41-31-631-4684; E-mail: fleck@izb.unibe.ch.

Running title: Rhp14 Mediated Mismatch Repair

SUMMARY

Rhp14 of *Schizosaccharomyces pombe* is homologous to human XPA and *Saccharomyces cerevisiae* Rad14, which act in nucleotide excision repair of DNA damages induced by ultraviolet light and chemical agents. Cells with disrupted *rhp14* were highly sensitive to ultraviolet light, and epistasis analysis with *swi10* (nucleotide excision repair) and *rad2* (Uve1-dependent ultraviolet light damage repair pathway) revealed that Rhp14 is an important component of nucleotide excision repair for ultraviolet light induced damages. Moreover, defective *rhp14* caused instability of a GT repeat, similar to *swi10*, and synergistically with *msh2* and *exo1*. Recombinant Rhp14 with an N-terminal hexahistidine-tag was purified from *E. coli*. Complementation studies with a *rhp14* mutant demonstrated that the tagged Rhp14 is functional in repair of ultraviolet radiation induced damages and in mitotic mutation avoidance. In band shift assays, Rhp14 showed a preference to substrates with mismatched and unpaired nucleotides. Similarly, XPA bound more efficiently to C/C, A/C and T/C mismatches than to homoduplex DNA. Our data show that mismatches and loops in DNA are substrate of nucleotide excision repair. Rhp14 is likely part of the recognition complex, but alone not sufficient for the high discrimination of nucleotide excision repair for modified DNA.

INTRODUCTION

In the course of nucleotide excision repair (NER)¹ of damaged bases, a preincision complex is assembled at the lesion. This complex contains XPA, RPA, XPC, hHR23B and TFIIH in humans, and homologous proteins in other eukaryotes (1–3). Subsequently, the damaged base is released from DNA in a 24–32 nucleotide long oligomer after dual incision by XPG, incising 3' to the lesion, and ERCC1-XPF, incising 5' to the lesion. Finally, repair is completed by resynthesis of the gap and ligation. It is not exactly known, whether one of the components of the preincision complex is the first damage recognition factor, or whether rather the entire preincision complex is required. Specific binding to damaged DNA has been reported for XPA, RPA, and XPC (4–11). However, the single proteins, as well as the complexes XPA-RPA and XPC-hHR23B exhibit only a moderate preference for damaged DNA. Thus, these factors likely contribute to recognition of damaged DNA, but alone might be not sufficient for a high discrimination.

NER is able to repair a variety of bulky DNA adducts, like (6–4) photoproducts and cyclobutane pyrimidine dimers induced by ultraviolet (UV) radiation, intrastrand crosslinks produced by *cis*-diamine-dichloroplatinum(II), and adducts formed by carcinogens such as benzo[*a*]pyrene diol epoxide (1, 12–14). In addition, nonbulky lesions such as methylated bases, and apurinic/apyrimidinic sites and to a low level, even G/A and G/G base-base mismatches are processed by NER (1, 12). In general, DNA that contains a lesion and some degree of helical distortion is more efficiently processed by NER than lesions without helical distortions or distortions alone (15–18).

Although NER incises mismatch-containing DNA rather poorly in vitro (12, 15–17), there is some evidence that NER factors have a function in correction of mismatched bases. Several mutations in the *S. cerevisiae* gene *RAD3*, encoding a homologue of human XPD, cause increased spontaneous mutation rates (19, 20). A mutated *mei-9* of *Drosophila melanogaster*, encoding a homologue of human XPF, results in increased postmeiotic segregation of genetic markers (21, 22). Elevated postmeiotic segregation frequencies are the consequence of non-repaired mismatches formed in heteroduplex DNA during meiotic recombination. In vitro mismatch correction is reduced in protein extracts of a *Drosophila mei-9* mutant (23). Mutations in the *Schizosaccharomyces pombe* NER genes *swi10* (ERCC1 homologue), *rad16* (XPF homologue), and *rhp14* (XPA homologue) cause a defect in repair of base-base mismatches, arising during vegetative growth and meiotic recombination (24).

This study aimed to extend the analysis on mismatch correction by NER factors. We report the characterization of the fission yeast *S. pombe* Rhp14 with respect to its function in DNA repair. A *rhp14* gene disruption mutant was constructed and tested for sensitivity to UV light and for stability of a GT repeat. In addition, recombinant Rhp14 was purified and analyzed for its capacity to bind to base-base mismatches and small loops.

EXPERIMENTAL PROCEDURES

S. pombe strains—*S. pombe* strains derived either from Ru39 (h^- *msh2::his3⁺* *his3-D1*) (25), SK15 (h^{90} *swi10::ura4⁺* *ura4-D18*) (26), OL455 (h^- *swi10::kanMX his3-D1 leu1-32 ura4-D18*)², sp217 (h^- *rad2::ura4⁺* *ade6-704 leu1-32 ura4-D18*) (27), OL142 (h^+ *cmb1::his3⁺* *his3-D1*) (28), Ru42 (h^- *exo1::ura4⁺* *ura4-D18*) (29, 30), or OL456 (h^- *rhp14::kanMX his3-D1 leu1-32 ura4-D18*; this study). The *ade6-485* mutation was described by Schär and Kohli (31), and *ade6-[(GT)₈-1397]* by Mansour *et al.* (32).

OL456 was constructed by transforming MAB031 (h^- *his3-D1 leu1-32 ura4-D18*) with a polymerase chain reaction (PCR) fragment containing the *kanMX* cassette flanked by sequences homologous to the 5' and 3' flanking sites of the *rhp14⁺* gene. The PCR fragment was obtained with primers DR14-5, 5'-

TCATTTCTAACAATAGCCATTCTCATTTTGGATTATTATTTGATTTTTTGAATTATCAT
ACTAGGGAAATAAAATAAAAAAAGTTCCTGACGCCAGCTGAAGCTTCGTAC-3' and

DR14-3b, 5'-

ACCCGATTTTTGAAGAAATCTGATAGCATTTACATTGAAAAAAGTTAGGTGTGCAAA
TCCAAAGTCTAAAAACAAAGACCATCGATGAATTCGAGCTCG-3'. PCR included 20

pmol of each of the two primers, 5 units of *Taq* polymerase, 0.15 mM each dNTP and ~50 ng pFA6a-kanMX6 (33) as template in a standard reaction buffer (Amersham Pharmacia Biotech). Conditions were: 5 min 94 °C; 3 cycles with 45 sec at 94 °C, 45 sec at 45 °C, 2 min at 72 °C; 30 cycles at 94 °C, 45 sec at 55 °C, 2 min at 72 °C, and a final 10 min step at 72 °C.

S. pombe media—*S. pombe* media YEA (yeast extract agar, complete medium), YEL (yeast extract liquid), MEA (malt extract agar, sporulation medium) and MMA

(minimal medium) were prepared as described (34, 35). EMM (Edinburgh minimal medium) without thiamine (36) was used for expression of *rhp14*, inserted in derivatives of pREP42 or pREP82 (see below). In these vectors *rhp14* is under the control of the *nmt* promoter, which is transcribed in the absence of thiamine (37, 38).

Expression and purification of [His]₆-Rhp14 from E. coli—The *rhp14*⁺ gene was amplified by PCR with primers ER14-5, 5'-CACCATATGGAAAATTCGTCAATTGTC-3' and ER14-3, 5'-GTGGGATCCTTAAATTTCCAGCTGCTCAA-3' and genomic *S. pombe* DNA as template in a standard reaction buffer containing 10 pmol of each of the primers, 0.1 mM each dNTP, 5 units of *Taq* polymerase (Amersham Pharmacia Biotech). Amplification was done by 5 min 94 °C, 30 cycles of 45 sec 94 °C, 45 sec 47 °C, 1 min 72 °C, and a final 10 min step at 72 °C. The PCR fragment was digested with *Nde*I/*Bam*HI and ligated with digested pET28c (Novagen). Plasmids containing *rhp14*⁺ fused to a hexahistidine- ([His]₆)-tag at the 5' end were identified by restriction digests and checked for mutations by DNA sequencing.

For expression, the plasmid pET28c-[His]₆Rhp14 was transformed into *E. coli* strain BL21/DE3. One liter LB medium containing kanamycin (30 mg/l) was inoculated with a 25-ml stationary phase culture and incubated at 37 °C until an A₆₀₀ of about 0.8 was reached. Isopropylthio-β-D-galactoside (1 mM) and ZnCl₂ (10 μM) were added and incubation continued for 4 h at 25 °C. Cells were harvested by 7 min centrifugation at 8300 × *g* and suspended in 30 ml buffer K (50 mM K₂HPO₄, pH 8.0, adjusted with KH₂PO₄ at room temperature, 100 mM KCl, 10% glycerol (v/v), 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol). Cells were shock frozen in liquid nitrogen and were allowed to thaw on ice overnight.

For preparation of crude protein extracts, cells were sonicated on ice by ten 30-sec intervals, with at least 2 min cooling on ice between each interval. Subsequently, the supernatant (116 mg protein in 24 ml) was separated by centrifugation for 20 min at $10,000 \times g$ and 4 °C. The supernatant was loaded at a flow rate of 2.3 ml/h on a Ni-NTA agarose column (Qiagen, $0.2 \text{ cm}^2 \times 6 \text{ cm}$) previously equilibrated with buffer K. After washing with buffer K, bound proteins were eluted by a 40-ml gradient of 0–70 mM imidazol in buffer K. [His]₆-Rhp14 started to elute at ~50 mM imidazol. Later fractions (~60–70 mM imidazol), containing [His]₆-Rhp14, but only low amounts of contaminating *E. coli* proteins, were pooled (3.2 mg in 4 ml), dialyzed against buffer K and loaded at a flow rate of 7 ml/h on a double-stranded DNA cellulose column (U.S. Biochemical Corp., $0.64 \text{ cm}^2 \times 4.7 \text{ cm}$). After washing with buffer K, bound proteins were eluted by a 50-ml gradient of 100–600 mM KCl in buffer K. Most of [His]₆-Rhp14 protein eluted between 100–160 mM KCl. In these fractions, no other proteins were detectable on 12% SDS polyacrylamide gels stained either with Coomassie blue (Fig. 4) or with silver nitrate (data not shown). Fractions were pooled and dialyzed against buffer K containing 50% (v/v) glycerol. Dialyzed fractions (1 mg protein in 1.8 ml) were stored in aliquots at –20 °C.

[His]₆-Rhp14 was detected by a Western blot according to the instructions of the manufacturer using mouse penta-His antibody (Qiagen) and horse radish peroxidase conjugated anti-mouse IgG (DAKO, Denmark) as secondary antibody.

Preparation of S. pombe crude protein extracts—*S. pombe* strains were grown to stationary phase in 1 liter YEL, harvested by centrifugation, washed with 15 ml buffer A (25 mM Tris-HCl (pH 7.5, adjusted at room temperature), 150 mM NaCl, 1 mM EDTA,

0.5 mM spermidine, 5 mM β -mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) and suspended in the same buffer. Aliquots (1 g cells/2 ml) were shock frozen in liquid nitrogen and stored at -70°C .

For preparation of crude protein extracts, cells were thawed on ice, mixed with an equal volume of glass beads and disrupted in a Fastprep FP 120 (Savant Instruments, Inc.) by eight 30 sec intervals, with 1 min cooling on ice between each interval. Proteins in the supernatant were removed from cell debris after 20 min centrifugation at 4°C .

Band shift analysis—Oligonucleotides were 5' end-labeled by T4 polynucleotide kinase (MBI Fermentas) in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and separated from unreacted ATP using Sephadex G25 (Amersham Pharmacia Biotech). Oligonucleotides were annealed with the complementary strands in 10 mM Tris-HCl (pH 8.0), 10 mM MgCl_2 , 80 mM NaCl by heating to 80°C and slow cooling to room temperature. Oligonucleotides were in the sequence context of either M13mp9 (39) or *ade6-485* (Fig. 1).

Band shift assays with *S. pombe* extracts ($\sim 50\ \mu\text{g}$ protein) were performed in $20\ \mu\text{l}$ reactions containing 20 fmol radiolabeled substrate, a 40-fold excess of unlabeled homoduplex DNA as competitor, 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 25 mM KCl, 0.01 mM ZnCl_2 , 0.5 mM dithiothreitol, 4 mM spermidine, and 10% glycerol (v/v). Band shift assays with $[\text{His}]_6\text{-Rhp14}$ (0.5-0.75 μg), purified from *E. coli*, were performed in $20\ \mu\text{l}$ reactions containing 40 fmol radiolabeled substrate, a 60-fold excess of unlabeled competitor, 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.01 mM ZnCl_2 , 0.5 mM dithiothreitol, 4 mM spermidine, 10% glycerol, and 1 μg bovine serum albumin. Band shift assays with purified XPA (0.4 μg) were performed in $20\ \mu\text{l}$ reactions, containing 20 fmol radiolabeled

substrate, a 40-fold excess of unlabeled homoduplex, 25 mM Tris-HCl (pH 7.5), 25 mM KCl, 0.01 mM ZnCl₂, 0.5 mM dithiothreitol, 4 mM spermidine, and 10% glycerol.

Reactions including *S. pombe* crude extracts were incubated for 20 min at 4 °C and subsequently loaded on 6% non-denaturing polyacrylamide gels. Reactions with purified [His]₆-Rhp14 or XPA were incubated for 30 min at 4 °C and loaded on 5% non-denaturing polyacrylamide gels. Electrophoresis was performed in 40 mM Tris-acetate (pH 7.5) at 90 V and 4 °C. XPA with an N-terminal [His]₆-tag was purified as described previously (5).

For quantification of DNA binding, gels were exposed to a phosphorimager and subsequently analyzed using ImageQuant software (Molecular Dynamics). The percentage of bound substrate was calculated from the intensity of shifted bands relative to the sum of bound and free radioactively labeled oligonucleotides. A reaction mix without protein was loaded on the gels to normalize the background level of radioactivity at the position of the shifted bands in protein containing reactions as well as to serve as a control for estimation of the total amount of substrates (see Fig. 6A, lane 1). A smear in the gels between free and bound substrates was usually detected when reactions contained either Rhp14 or XPA. The smear likely reflects loss of protein-DNA interaction during electrophoresis and was not included in the calculation.

Construction of plasmids for expression of Rhp14 in S. pombe—In a first step pREP42 and pREP82 (38) derivatives were constructed, which contain the polylinker 5'-CATATGGCCATGGCTAGCCCTCGAGGTCGACATGCATGGATCCCCGGG-3', harboring the restriction sites *NdeI-NcoI-NheI-XhoI-SalI-NsiI-BamHI-SmaI*, resulting in pREP42L and pREP82L. Subsequently, the 0.9 kb *NcoI-BamHI* fragment from pET28c-

[His]₆Rhp14 was ligated with digested pREP42L or pREP82L, resulting in p[His]₆Rhp14 plasmids. In these plasmids, the *rhp14* gene is under control of the *nmt* promoter and, as in pET28c-[His]₆Rhp14, expressed as a fusion protein with an N-terminal His-tag. pRhp14 derivatives, expressing Rhp14 without His-tag, were obtained after digestion of p[His]₆Rhp14 plasmids with *Nde*I and religation. Plasmids were transformed into *S. pombe* strains PRS69 (*h⁻ ura4-D18 ade6-485*) and OL549 (*h⁻ rhp14::kanMX ura4-D18 ade6-485*). For expression of Rhp14, transformants were propagated in EMM liquid medium supplemented with adenine (100 mg/l).

UV sensitivity tests—For quantitative determination of survival of UV irradiated cells (Fig. 2), strains were grown in YEL to stationary phase at 30 °C and appropriate dilutions were plated on YEA. Cells were irradiated in a UV Stratalinker (Stratagene), and incubated for 5 days at 30 °C. Survival was determined from the number of cells able to grow to colonies relative to unirradiated controls.

For the complementation test (Fig. 5A), strains were grown to stationary phase in liquid EMM supplemented with adenine (100 mg/l). 10 µl of serial 1:10 dilutions were dropped on EMM + adenine. Plates were UV irradiated (20-100 J/m²) in an UV Stratalinker (Stratagene) and incubated together with an unirradiated control plate for 3 days at 30 °C.

Determination of mitotic mutation rates—Reversion rates per cell division were calculated from the median number of Ade⁺ per total cell number of cultures (40). For fluctuation tests, either *ade6-(GT)₈* or *ade6-485* was used. The *ade6-(GT)₈* allele represents a (GT)₈ repeat, which was constructed by insertion of seven GT units at an existing GT site (32). Nine colonies grown on YEA were each inoculated in 5-ml YEL

and grown to stationary phase. Appropriate amounts were plated on MMA for selection of revertants and on MMA supplemented with adenine for determination of cell titers. Plates were incubated for 7 days at 30 °C.

Determination of reversion rates of 485 (a C to G transversion) was carried out with wild type (h^- *ura4-D18 ade6-485*) containing empty vector (pREP42L or pREP82L) and with a *rhp14* mutant (h^- *rhp14::kanMX ura4-D18 ade6-485*) transformed with empty vector, pRhp14, or p[His]₆Rhp14. Nine colonies, grown on EMM supplemented with adenine, were inoculated in 5 ml liquid EMM (+ adenine) and grown to stationary phase. Cells were harvested by centrifugation, suspended in 600 µl 0.85% NaCl and plated on EMM. Appropriate dilutions were plated on EMM (+ adenine) for cell titer determination. Plates were incubated for 12 days at 30 °C. For each strain background, experiments were carried out at least three times.

Identification of deletions and insertions in the (GT)₈ repeat—Mutational changes in the (GT)₈ repeat were determined by DNA sequencing of PCR products and by visual inspection of colony colors as described (32). Revertants with deletions of GT units, which retain the open reading frame of *ade6* [(GT)₄ or (GT)₇], form white colonies, while revertants with insertion of four nucleotides [(GT)₁₀] form pink colonies. Other repeat tract changes were not identified by DNA sequencing (32; and data not shown).

RESULTS

Rhp14 is involved in the NER pathway for UV induced damages—The *S. pombe* *rhp14* gene was identified by the *S. pombe* Genome Sequencing Project (http://www.sanger.ac.uk/Projects/S_pombe/). The deduced amino acid sequence shows 33% identity to human XPA and 39% identity to Rad14 of *S. cerevisiae*, which are involved in the damage recognition step of NER (1-3). A *rhp14* gene disruption strain was constructed as described in “Experimental Procedures” and was tested for cell survival after irradiation with different doses of UV light (Fig. 2). *rhp14* cells were highly sensitivity to UV, to a similar extent as *swi10*, defective in the NER 5' endonuclease (26, 41, 42), and somewhat more affected than *rad2* cells, defective in the second, Uve1-dependent pathway for repair of UV damages in *S. pombe* (43, 44). The *rhp14 swi10* double mutant showed similar sensitivity to UV as either single mutant, while the *rhp14 rad2* double mutant was clearly more sensitive (Fig. 2). Thus, Rhp14 is an important factor of NER of UV induced damages, but not component of the Uve1-dependent pathway.

GT repeat stability is affected in the rhp14 mutant—The long-patch mismatch repair (MMR) system of *S. pombe* efficiently corrects base-base mismatches, except C/C, as well as one to several unpaired nucleotides (24, 25, 30–32, 35, 45). A genetic test system was developed that allows measuring instability of GT repeats (32). Loss of MMR by a mutation in *msh2*, *msh6*, or *pms1* resulted in dramatically increased instability of GT repeats. Exo1 is likely involved in MMR-dependent repair of base-base mismatches (29, 30), but has only a minor and rather MMR independent function in GT repeat stability (32).

It has been previously shown that NER factors of *S. pombe* are involved in MMR independent short-patch repair of base-base mismatches arising during meiotic recombination and vegetative growth (24). Here we tested the consequences of mutations in the NER factors *rhp14* and *swi10* on Ade⁺ reversions of a (GT)₈ repeat introduced into the *ade6* gene. In addition, epistasis analysis, including *msh2* and *exo1*, was performed (Table I). *ade6*-(GT)₈ originated from an insertion of seven GT units at an existing GT site, and thus represents a frame shift mutation causing adenine auxotrophy (32). Reversions to Ade⁺, which restore the reading frame, can either occur by deletions of 2 or 8-bp (1 or 4 GT units) or by insertions of 4-bp (two GT units).

In repair-proficient wild type a reversion rate of 3×10^{-9} was measured (Table I). With a 1.8×10^4 -fold increased rate, the (GT)₈ repeat was highly destabilized in the *msh2* mutant, as expected from the previous study (32). Reversion to Ade⁺ occurred 12-times more frequently in *exo1* cells than in wild type. A similar increase was found with the NER mutants *rhp14* and *swi10*. Compared to respective single mutants, a further increase of reversion rates was found in the double mutants *msh2 rhp14*, *msh2 swi10*, *rhp14 exo1*, and *swi10 exo1*, but not with *rhp14 swi10*. Thus, Rhp14 and Swi10 act in the same pathway for maintaining GT repeat stability, and independent of Msh2 and Exo1.

The relative distribution of deletions versus insertions of GT units can be easily determined by inspection of the colony color of revertants (32). As confirmed by DNA sequencing, revertants forming white colonies contain (GT)₄ or (GT)₇ repeats (deletion of 8 and 2-bp, respectively), while revertants forming pink colonies contain a (GT)₁₀ repeat (insertion of 4-bp). In wild type and the *exo1* mutant, most of the Ade⁺ revertants

were pink and thus originated from 4-bp insertions (Table II). In contrast, almost all of the revertants in *msh2* background produced white colonies (Table II), and sequencing of 11 of them exclusively identified a (GT)₇ repeat and thus a 2-bp deletion (32). In *rhp14* and *swi10* mutants, about 70% of revertants formed pink colonies. Thus, (GT)₈ mainly reverted to Ade⁺ by insertions of 4-bp (Table II). Among the white revertants, both (GT)₄ and (GT)₇ repeats were identified by sequencing (data not shown). Thus, the mutation spectra of *rhp14* and *swi10* are similar to that of wild type.

C/C mismatch binding by Cmb2 is not affected in rhp14 cells—Since Rhp14 of *S. pombe* is a component of MMR-independent repair of mismatched DNA (24, and this study), it is conceivable that Rhp14 specifically recognizes mismatched or unpaired nucleotides. The defect in correction of base-base mismatches in NER mutants is most pronounced for C/C mismatches, which are not substrate of MMR (24, 30, 31). The Cmb1 protein was recently identified as a recognition factor for C/C and other cytosine containing mismatches (28). In crude protein extracts of a *cmb1* disruption strain, binding to cytosine-containing mismatches was abolished, with the exception of C/C, where still some binding was detected. The gene encoding the second C/C binding activity, Cmb2, was not yet identified. Therefore, we started our mismatch-binding studies on Rhp14 with the analysis of the C/C binding capacity of crude extracts of *rhp14* mutants, either additionally mutated in *cmb1* or not (Fig. 3). In crude extracts of wild-type cells, specific binding to C/C and T/C was detectable. In *cmb1* cells, binding to C/C by Cmb2 remained. Binding by either Cmb1 or Cmb2 was not affected in *rhp14* extracts, and C/C binding by Cmb2 was still present in extracts of the *rhp14 cmb1* double mutant. Thus, Cmb2 is not encoded by the *rhp14* gene.

Purification of recombinant Rhp14 from E. coli—The *rhp14* gene was overexpressed in *E. coli* BL21/DE3 as a fusion protein with an N-terminal [His]₆-tag as described in “Experimental Procedures”. [His]₆-Rhp14 was purified by chromatography through binding on Ni-NTA agarose and double-stranded DNA-cellulose columns (see under “Experimental Procedures”). [His]₆-Rhp14 started to elute from the Ni-NTA agarose column in the presence of ~50 mM imidazol together with five additional peptides in considerable amounts. In later fractions, which eluted by ~60-70 mM imidazol, [His]₆-Rhp14 was still present in high amounts, while the *E. coli* proteins were at much lower concentrations. Rhp14 in the later fractions was successfully separated from the remaining proteins using a double-stranded DNA cellulose column (Fig. 4A). The *E. coli* proteins were detected in the flow through fractions, while most of Rhp14 bound to the column and eluted in the presence of ~100-160 mM NaCl. That the purified protein was indeed [His]₆-Rhp14 was proved by a Western using a His-tag specific antibody as probe (Fig. 4B).

[His]₆-Rhp14 is active in DNA repair in vivo—To test whether the [His]₆-tag interferes with the function of Rhp14, complementation of DNA repair defects caused by mutated *rhp14* was studied. Therefore, an *S. pombe rhp14* mutant strain was transformed with plasmids derived from pREP42L either containing *rhp14* (pRhp14) or tagged *rhp14* (p[His]₆Rhp14). As controls wild-type and *rhp14* strains were transformed with the empty vector pREP42L. Both, complementation of UV sensitivity and of increased mitotic mutation rates were tested.

The *rhp14* mutant containing pREP42L (Fig. 5A) or pREP82L (data not shown) was extremely sensitive to UV light. When transformed either with pRhp14 or p[His]₆Rhp14,

survival rates were retained to ~40% of that of wild-type cells. That the defect in damage repair was not completely compensated is likely due to negative effects of overexpressed Rhp14, which is under control of the strong *nmt* promoter (37, 38). However, because no difference to cells containing untagged Rhp14 was found, it is likely that [His]₆-Rhp14 is fully active in UV damage repair in vivo. In addition, increase of the reversion rate at the *ade6-485* locus, caused by mutated *rhp14*, was completely compensated by p[His]₆Rhp14 (Fig. 5B). Thus, [His]₆-Rhp14 is also functional in mismatch correction.

Binding of Rhp14 to base-base mismatches and DNA loops—Rhp14 is based on its homology to XPA and Rad14, likely involved in the recognition step of NER, and might therefore have some preference in binding to modified over homoduplex DNA. In addition, Rhp14 is required for mismatch correction (24; and Fig. 5B) and to some degree for GT repeat stability (Table I). Therefore, we tested whether purified Rhp14 can specifically bind to substrates containing base-base mismatches or unpaired nucleotides by a band shift assay. The oligonucleotides were in the sequence context of *ade6-485* (see Fig. 1), which is known to be substrate of NER in vivo (24). Substrates containing C/T, C/C or C/Δ mismatches were about two-times better bound than homoduplex DNA (Fig. 6). A similar affinity was found for T/G and T/T (data not shown), while binding to C/A was only slightly stronger and not significantly different to homoduplex binding.

Rhp14 also showed increased affinity to substrates containing loops with one, two or four nucleotides (Fig. 7). Tendentiously, the loops with four unpaired nucleotides,

(GTGT and ACAC) were stronger bound than loops with two (GT and AC) or one unpaired nucleotide.

Binding of human XPA to base-base mismatches—Since NER in *S. pombe* is involved in MMR-independent mismatch correction (24) and because Rhp14 showed some specific affinity to mismatched DNA (Fig. 6), we were interested to know whether mismatch processing by NER is a general feature in eukaryotes. Therefore, we tested the ability of human XPA to recognize base-base mismatches (Fig. 8). Compared to homoduplex DNA, increased binding was observed with substrates containing a C/C, A/C or T/C mismatch. These data indicate that mismatches are specifically recognized by XPA. Similar to Rhp14, only a slightly higher affinity than to homoduplex DNA was detected.

DISCUSSION

This study reports the characterization of Rhp14 with respect to its function in repair of UV damages and mismatched DNA, as well as its ability to specifically bind to base-base mismatches and small insertion/deletion loops. Rhp14 is, based on its homology to human XPA and *S. cerevisiae* Rad14, likely involved in the recognition step of NER.

In the initial experiment we found that the *rhp14* mutant was as sensitivity to UV light as the *swi10* mutant, defective in the NER 5' endonuclease, and UV sensitivity was not further increased in the *rhp14 swi10* double mutant (Fig. 2). Thus, Rhp14 plays indeed an important role in NER. Consistently, the *rhp14 rad2* mutant was more sensitive to UV than either single mutant, showing that Rhp14 is not involved in the Uve1-dependent pathway.

Since NER factors in *S. pombe* have an MMR-independent role in repair of base-base mismatches (24), we analyzed the effects of mutated *rhp14* and *swi10* on stability of a GT repeat, a common type of microsatellite in eukaryotic genomes. Reversions of the (GT)₈ repeat occurred with high frequency in *msh2* cells, defective in MMR (Table I). Compared to wild type, about 12-14 fold increased reversion rates were observed with *exo1*, *rhp14* and *swi10* mutants. Epistasis analysis with double mutants revealed that Rhp14 and Swi10 act in the same pathway for maintaining GT repeat stability and distinct from Msh2 and Exo1. The distribution of insertions and deletions of GT units was similar to that of wild type (Table II), while in the *msh2* mutant, most reversions occurred by deletion of 2-bp (32; and Table II). The data confirm previous studies, which showed that MMR plays a dominant role in stability of GT repeats and other microsatellites (32, 46–49) and further suggest that NER contributes to maintaining the

tract length of a (GT)₈ repeat in *S. pombe*. Thus, NER might serve as a backup system for stabilization of microsatellites. Consistently with the genetic analysis, Rhp14 showed an about 2-fold preference to substrates with either a GT, (GT)₂, AC or (AC)₂ loop (Fig. 7). Such loops can be formed in GT repeats by strand slippage during replication.

DNA-binding capacity was studied with Rhp14 containing an N-terminal [His]₆-tag. To ensure that the tag did not interfere with the function of the protein, we tested complementation of DNA repair defects caused by mutated *rhp14*. Both UV sensitivity and increased *ade6-485* reversion rates were complemented to the same degree by [His]₆-tagged Rhp14 and by untagged Rhp14 expressed on a plasmid (Fig. 5). Thus, [His]₆-Rhp14 is functional in vivo.

In band shift assays the Rhp14 protein showed preferential binding to substrates containing base-base mismatches or loops with unpaired nucleotides (Fig. 6 and 7). Our recent study revealed that mutated *rhp14* and *swi10* caused elevated mitotic mutation rates of the *ade6-485* allele (a C to G transversion), likely due to the failure to repair C/C mismatches (24). In addition, short-patch repair of C/C mismatches formed during meiotic recombination was strongly affected in the NER mutants. We found no significant difference in binding of Rhp14 to C/C and other types of mismatches (Fig. 6). Thus, the observation that NER predominantly repairs C/C is rather due to the fact that C/C mismatches are not processed by MMR, and likely not a consequence of preferential recognition of C/C. Consistently, in the absence of MMR, NER can also process other types of mismatches (24). However, it should be noted that *S. pombe* contains two activities, Cmb1 and Cmb2, which recognize C/C (28). Cmb1 also binds to other types of cytosine-containing mismatches, while Cmb2 exclusively binds to C/C.

Generally, only weak effects on DNA repair were found in a *cmb1* mutant³, which might be due to redundant functions with Cmb2, whose gene was not yet identified. Since binding to C/C remained in the protein extract of a *rhp14 cmb1* mutant, the *rhp14* gene does not encode for Cmb2 (Fig. 3). The role of Cmb1 and Cmb2 in DNA repair is not yet understood. One possibility is that they act as accessory factors in DNA repair.

Binding of Rhp14 to mismatches and loops was about 2-fold stronger than to homoduplex. A similar specific affinity was found for binding to C/C, A/C and T/C mismatches by XPA (Fig. 8). A recent study revealed that XPA shows an about 2-fold higher affinity to (6-4) photoproducts than to unmodified homoduplex (10). The preference for damaged and mismatched DNA might be due to local melting of the double helix. In fact, XPA binds with similar affinities to substrates containing either a bubble with three mispaired nucleotides or a benzo[a]pyrene adduct (14). It was suggested that XPA recognition requires sites in DNA with disrupted base-pairing. To serve as recognition signal for XPA, the helical distortion should be in the context of duplex DNA, since single-stranded DNA is not better bound than double-stranded unmodified DNA (14).

The observation that NER is able to correct mismatches in *S. pombe* suggests that mismatches are actively recognized by NER factors. Rhp14 likely contributes to discrimination between modified and unmodified DNA, but for efficient recognition additional factors are likely required. An interesting question is whether correction of mismatches and loops by NER is a special situation in *S. pombe* and may be in a few other organisms, or whether it is a general feature of eukaryotes. Only a limited set of

data are available so far supporting the latter possibility, and there are clearly more experiments necessary to answer this question.

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¹ The abbreviations used are: NER, nucleotide excision repair; UV, ultraviolet; Rhp14, Rad14 homologue pombe; [His]₆, hexahistidine; PCR, polymerase chain reaction; MMR, long-patch mismatch repair; bp, base pair(s).

² C. Kunz and O. Fleck, manuscript in preparation.

³ C. Kunz, K. Zurbriggen and O. Fleck, manuscript in preparation.

FIGURE LEGENDS

FIG. 1. **Substrates used to test DNA binding by band shift assays.** Substrates were in the context of *ade6-485* (31). Complementary oligonucleotides were combined by annealing resulting in double-stranded DNA with defined base-base mismatches, unpaired nucleotides, or correctly paired bases at the position X/Z, the site of the 485 mutation (a C to G transversion).

FIG. 2. **UV sensitivity of NER and *rad2* mutants.** Strains were irradiated with the indicated UV doses and cell survival was determined after five days of growth by the ability of cells to form colonies. Data are mean values and standard deviations from three experiments. Strains were wild type (●), *rad2* (△), *rhp14* (▲), *swi10* (◆), *rhp14 swi10* (○), and *rhp14 rad2* (■).

FIG. 3. **Mismatch binding in crude extracts of *S. pombe* strains.** Specific binding to C/C and T/C is detectable in extracts from wild type and *rhp14* cells (marked with an arrow). Deletion of *cmb1* abolished binding to T/C, but not of binding to C/C by Cmb2. This activity is also present in *rhp14 cmb1* extracts, demonstrating that Cmb2 is not encoded by *rhp14*. Oligonucleotides were in the M13mp9 context (39). Conditions of the band shift assay are described in "Experimental Procedures".

FIG. 4. **Purification of [His]₆-Rhp14 from *E. coli*.** A, Coomassie-stained 12% SDS polyacrylamide gel containing fractions passed through a double-stranded DNA cellulose column. The position of [His]₆-Rhp14 is marked by an arrow. M, broad range

marker (Bio-Rad) with numbers presenting sizes in kDa. Load, sample of the pooled fractions of proteins that eluted from the Ni-NTA column and that were loaded on the double-stranded DNA cellulose column. FT, flow through, containing proteins not bound to the column. *B*, Western blot of samples containing pooled fractions 5-10, shown in *A*. Different amounts of protein were used as indicated and were probed with penta-His antibody. *E. coli*, crude extracts of the *E. coli* strain BL21/DE3 not containing Rhp14.

FIG. 5. **[His]₆-tagged Rhp14 is active in DNA repair vivo.** *A*, Complementation of UV sensitivity of defective *rhp14* by plasmids expressing Rhp14 or [His]₆-tagged Rhp14. Wild type and *rhp14* strains containing the indicated plasmids were serially diluted and drops containing $\sim 10^5$, 10^4 , 10^3 and 100 cells were spotted on EMM plates. Plates were irradiated with 0, 20, 50 or 100 J/m² and were incubated for 3 days at 30 °C. Shown are the unirradiated control and the plate irradiated with 50 J/m². *B*, Complementation of the mutator effect of defective *rhp14* by plasmids expressing Rhp14 or [His]₆-tagged Rhp14. Wild type and *rhp14* strains containing the indicated plasmids were tested for reversion rates of the *ade6* allele 485. Columns represent mean values of reversion rates to Ade⁺ per 10⁹ cell divisions with standard deviations.

FIG. 6. **Binding of Rhp14 to mismatched DNA.** *A*, Binding of Rhp14 to homoduplex DNA and to the mismatches C/A, C/T, C/C and C/Δ was tested by a band shift assay as described in “Experimental Procedures”. In the first lane, a reaction mix containing C/Δ substrate without Rhp14 was loaded. Substrates were in the *ade6-485* context (Fig. 1). The position of the Rhp14-DNA complex is indicated by an arrow. *B*, Quantitative

expression of DNA binding by Rhp14. Columns represent the mean percentages of bound substrates with standard deviations, which were calculated from three band shift gels.

FIG. 7. **Binding of Rhp14 to DNA loops.** A, Binding of Rhp14 to homoduplex DNA ($\Delta:\Delta$) and to substrates containing one (Δ/C , Δ/G , Δ/A , Δ/T), two (GT/Δ , Δ/AC) or four [$(GT)_2/\Delta$, $\Delta/(AC)_2$] unpaired nucleotides. Substrates were in the *ade6-485* context (Fig. 1). The position of the Rhp14-DNA complex is indicated by an arrow. B, Quantitative expression of DNA binding by Rhp14. Columns represent the mean percentages of bound substrates from three band shift gels with standard deviations.

FIG. 8. **Binding of human XPA to mismatched DNA.** Band shift assays were performed as described in "Experimental Procedures". Substrates were in the M13mp9 context (39). The position of the XPA-DNA complex is marked by an arrow. Percentage of binding is given below the gel as average of two experiments.

TABLE I

Reversion rates of the (GT)₈ repeat

Reversion rates of the (GT)₈ repeat in the *ade6* gene (32) were determined as described in “Experimental Procedures”. Reversions can occur by deletion of 2- or 8-bp or by insertion of 4-bp (see Table II).

Relevant genotype	Reversion rate ^a	Fold increase ^b
Wild type	$3.0 \pm 1.1 \times 10^{-9}$	1
<i>msh2</i>	$5.5 \pm 1.5 \times 10^{-5}$	1.8×10^4
<i>exo1</i>	$3.5 \pm 0.9 \times 10^{-8}$	12
<i>rhp14</i>	$3.5 \pm 0.7 \times 10^{-8}$	12
<i>swi10</i>	$4.2 \pm 1.9 \times 10^{-8}$	14
<i>msh2 rhp14</i>	$3.2 \pm 0.6 \times 10^{-4}$	1.1×10^5
<i>msh2 swi10</i>	$3.1 \pm 1.3 \times 10^{-4}$	1.0×10^5
<i>rhp14 exo1</i>	$1.3 \pm 0.6 \times 10^{-7}$	43
<i>swi10 exo1</i>	$2.5 \pm 0.0 \times 10^{-7}$	83
<i>rhp14 swi10</i>	$4.2 \pm 0.4 \times 10^{-8}$	14

^a Numbers are mean values of at least three independent experiments with standard deviations.

^b Fold increase relative to wild type.

Table II

Distribution of deletions and insertions in the (GT)₈ repeat

Tract changes in the (GT)₈ repeat were determined by DNA sequencing and by inspection of the colony color of revertants (32). Ade⁺ revertants that form pink colonies contain insertions of 4-bp, while revertants forming white colonies resulted from deletions of either 2- or 8-bp in the (GT)₈ repeat.

Relevant genotype	% Deletions (−2 or −8 bp)	% Insertions (+4 bp)
Wild type	29	71
<i>msh2</i>	98	2
<i>exo1</i>	22	78
<i>rhp14</i>	33	67
<i>swi10</i>	28	72
<i>msh2 rhp14</i>	99	1
<i>msh2 swi10</i>	<99	>1
<i>rhp14 exo1</i>	36	64
<i>swi10 exo1</i>	44	56
<i>rhp14 swi10</i>	28	72

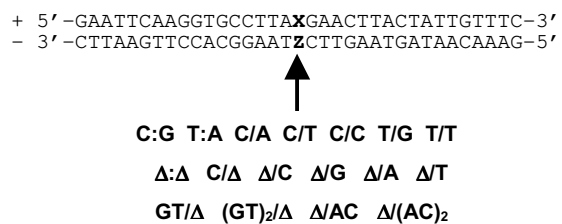


Figure 1

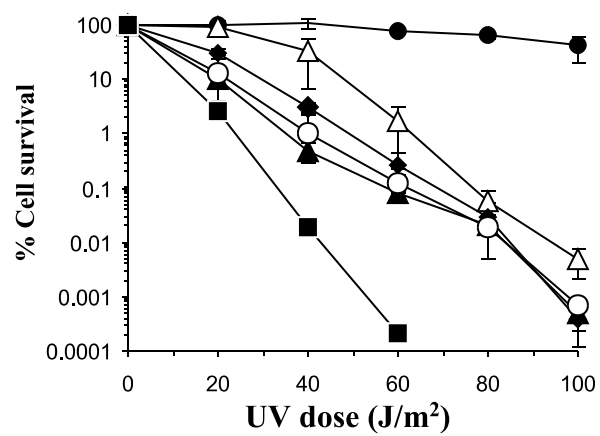


Figure 2

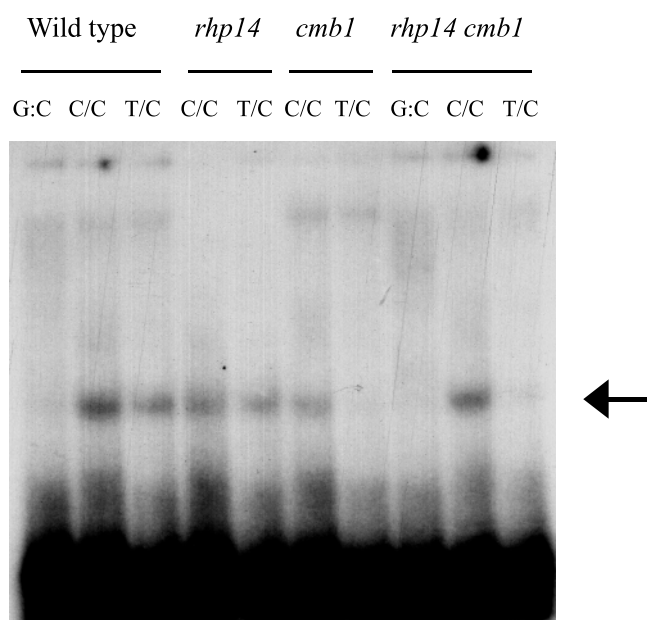


Figure 3

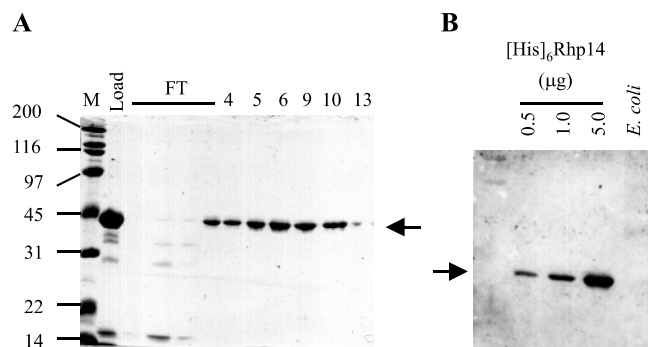


Figure 4

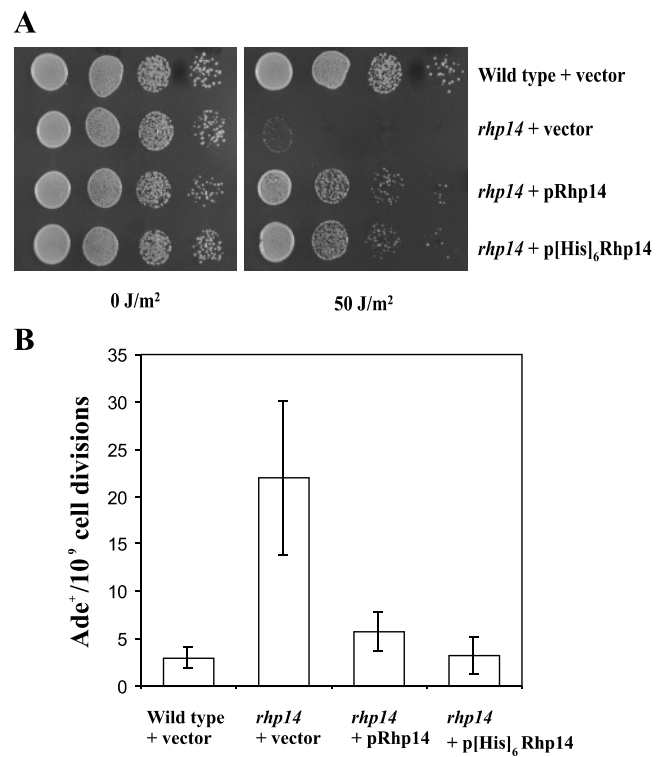


Figure 5

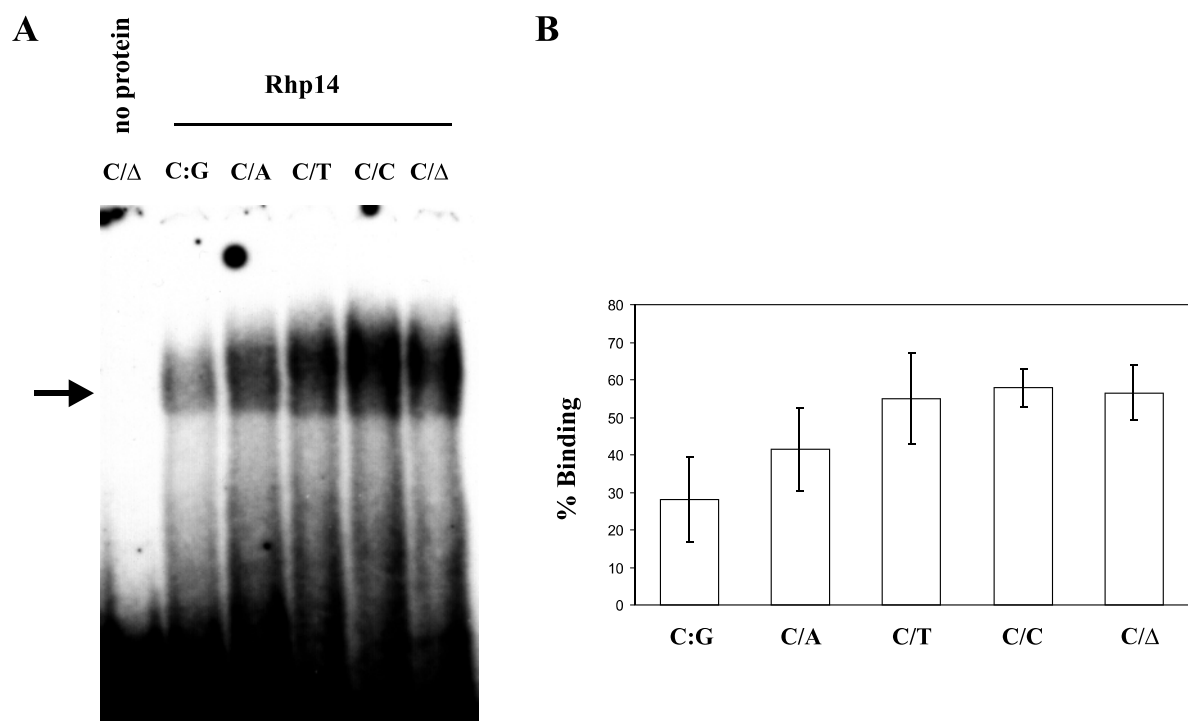


Figure 6

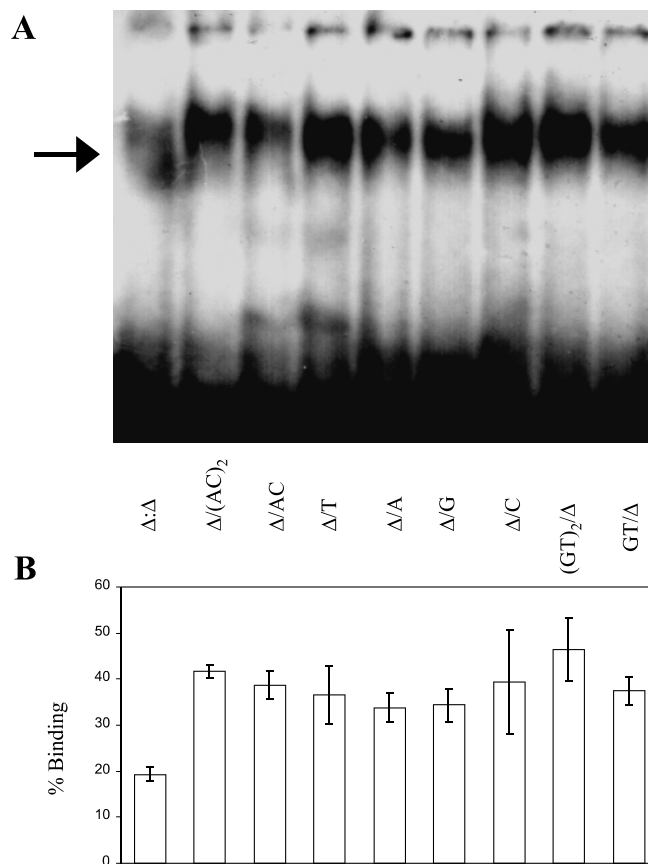


Figure 7

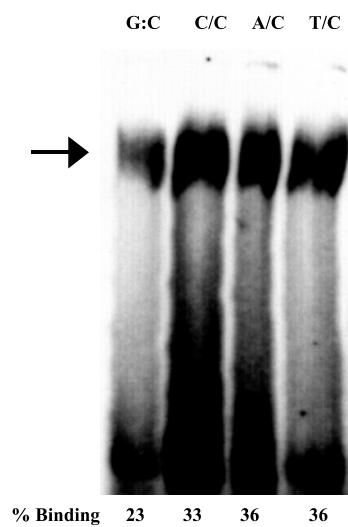


Figure 8